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Uptake of *Vibrio anguillarum* vaccine by *Artemia salina* as a potential oral delivery system to fish fry

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An ELISA to detect *Vibrio anguillarum* antigen in *Artemia salina* was developed. This assay was then used to determine the time course of antigen uptake by *Artemia* during incubations with *Vibrio* vaccine with a view to using *Artemia* as a vaccine carrier for oral vaccination of fish fry. The rate of uptake of vaccine by *Artemia* was shown to be concentration dependent. At a dose of $1{\cdot}5 \times 10^7$ cfu ml^{-1}, 1 mg wet weight of *Artemia* took up 1×10^5 cfu within 30 min and reached maximum of 6×10^6 cfu after 60 min. Thus each *Artemia* appears to have taken up approximately $1{\cdot}2 \times 10^5$ cfu. Comparison of vaccine formulations indicated that uptake was consistently higher with formalised than with heat-treated vaccine.

Key words: vibrio, vaccine, artemia.

I. Introduction

The benefits of producing an effective orally administered fish vaccine have long been recognised but no method reported to date has shown satisfactory, reproducible levels of protection. Kawai & Kusada (1985) for example found that for ayu (*Plecoglossus altivelis*), the level of protection against vibrosis resulting from feeding with formalin-killed whole-cell *Vibrio anguillarum* decreased with time. In a similar study, Kawano *et al.* (1984) found that protection of ayu against vibriosis continued for less than 60 days when the vaccine was administered orally, but for more than 113 days when administered by immersion.

Oral vaccination may be less effective than injection or immersion because of inadequate antigen uptake in the digestive tract, or because the antigen is broken down in the acid environment in the gut of the fish. Johnson and Amend (1983) proposed that protecting antigens from gastric secretions might improve the efficacy of orally administered vaccine since they discovered that when the antigen was presented by anal intubation, effective immunisation was achieved.

Potential problems of low palatability because of vaccine treatment, and loss of antigenicity during digestion can apparently be overcome by incorporating the vaccine into live food prey. It has been demonstrated that juvenile ayu can acquire protective immunity against vibriosis by feeding water flea

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Brachionus plicatilis which have accumulated the *Vibrio* antigens (Kawai *et al.*, 1989).

As a first step to investigating levels of protection achieved by feeding vaccine treated *Artemia* to fish fry, an ELISA was adapted to measure antigen uptake by *Artemia* after varying incubation times.

II. Materials and Methods

BACTERIA

Cultures of *V. anguillarum* strain 8587, supplied by Wellcome Biotech were grown in Tryptic soy broth (TSB Difco, U.K.) with no salt added, for 18 h at 22° C. Cultures were used for vaccine production, for ELISA standards and for the standard curves with colony forming units (cfu) against optical density.

PRODUCTION OF ANTISERUM

The antiserum was raised in a New Zealand white rabbit. The rabbit was injected twice subcutaneously, initially with 10^9 *V. anguillarum* strain 8587 ml^{-1} in complete Freund's adjuvant. The second injection was followed after a further 3 weeks by an intravenous boost of 10^9 cfu *V. anguillarum* ml^{-1}. Ten days later the rabbit was bled out.

PURIFICATION OF IgG FROM THE ANTISERUM

The IgG was purified from the antiserum by sodium sulphate (Na_2SO_4) precipitation. Sodium sulphate was added to 2 ml antiserum at 14% w/v and placed in a water bath at 25° C for 5 min. After centrifuging at 12 000 *g*, the supernatant was removed and the pellet was washed twice with 14% Na_2SO_4 at 25° C. The precipitate was dissolved in 2 ml phosphate buffered saline (PBS 0·02 M phosphate, 0·15 M NaCl, pH 7·4) at 25° C. The protein concentration was determined spectrophotometrically at 280 nm using an LKB Ultraspec 11. It is assumed that an optical density (O.D.) of 1·4 equates to 1 mg ml^{-1} protein and that this relationship is linear (Hudson and Hay, 1976)

The antibody was prepared for conjugation with horseradish peroxidase (HRP) by dialysing against three 2 l changes of sodium bicarbonate buffer (0·01 M, pH 9·5). Antibody for coating ELISA plates was prepared by making a solution of 3·6 mg ml^{-1} purified IgG in PBS, adjusted to pH 2·1 with hydrochloric acid. This was stirred for 30 min and then the pH was adjusted to 7 by the addition of solid Tris (hydroxymethyl) aminomethane (Trizma® Base, Sigma Chemical Co., Poole, Dorset, U.K.).

ACTIVATION OF HRP

Activation of horseradish peroxidase (HRP) was performed by dissolving 5 mg HRP (Sigma type VI, Sigma) in 1 ml freshly prepared bicarbonate buffer (0·3 M, pH 8·1). To this was added 0·1 ml flurodinitrobenzene. This was mixed gently for 1 h in an orbital incubator at room temperature, after which was added 1 ml, 0·05 M sodium iodate ($NaIO_4$) in distilled water. The solution was

mixed for 30 min at room temperature and then 1 ml 0·16 M ethylene glycol in distilled water was added. This was mixed for 1 h at room temperature and the solution was dialysed against bicarbonate buffer (0·01 M, pH 9·5) at 4° C, using three changes each of 2 l.

CONJUGATION

This was performed by the method of Wilson and Nakane (1978). The purified rabbit anti-*Vibrio* antibody (5 mg) was added to 3 ml activated HRP and mixed gently for 2–3 h at room temperature. To this was added 5 mg sodium borohydride ($NaBH_4$) and the solution was left overnight at 4° C. It was then dialysed against PBS at pH 7·0. The ratio at 403/280 nm was determined spectrophotometrically, a ratio of between 0·3 and 0·6 indicating successful conjugation.

ELISA

The assay was performed on Nunc-immuno Module Polysorp plates. These were coated with acid treated antibody at a concentration of 10 μg ml^{-1} in 0·05 M bicarbonate buffer containing 0·05% Merthiolate® (Sigma). The plate was incubated overnight at 4° C, or for 3 h at 30° C, in a sealed box. After incubation it was washed three times with a low salt wash buffer containing 0·02 M Tris, 0·38 M NaCl, 0·05% Tween 20 and 0·01% Merthiolate®, pH 7·4. The plate was tapped dry and post coated by adding 300 ml 1% bovine serum albumin (BSA) to each well. This was left at room temperature for 30 min and then washed once with low salt wash before application of the antigen. Bacterial standards containing known concentrations of antigen were prepared and applied to the plate along with the samples. To prepare standards, bacteria were grown on tryptic soya agar (TSA) plates without added salt for 1 week at 21° C. Plates were washed with 10 ml PBS into a 20 ml glass universal bottle. Samples were heat killed in a water bath at 60° C for 60 min and then centrifuged at 1000 *g*, washing twice with 10 ml PBS. The O.D. at 610 nm was determined and the solution was diluted with PBS to give an O.D. of 0·38, representing 10^8 cfu ml^{-1} (see Results). Samples were diluted with antigen diluent buffer (PBS with 0·01% Merthiolate® and 0·1% Tween 20) to give a range of concentrations from 10^7 to 10^2 cfu ml^{-1}. After applying 100 μl of each sample in duplicate to each well, it was incubated overnight at 4° C in a sealed box.

After incubation the plate was washed three times with high salt wash, containing 0·02 M Tris, 0·05M NaCl, 0·1% Tween 20 and 0·01% Merthiolate®, pH 7·7.

The final wash was preceded by a 5-min soak. The plate was tapped dry and then the conjugate was added (100 μl/well) at a dilution of 1/200 in PBS with 10% (v/v) normal sheep serum. This was incubated for 90 min at room temperature and then washed three times with high salt wash buffer with one 5-min soak. The plate was tapped dry and 100 ml chromogen (42 mM 3,3′,5,5′-tetra methylbenzidine dihydrochloride, Sigma) in substrate buffer was added to each well. The chromogen (42 mM) in 2 M acetic acid was added (120 μl) to

12 ml substrate buffer (0·1 M citric acid, 0·1 M sodium acetate, pH 5·4 containing 0·33% H_2O_2). The reaction was terminated after 10 min by adding 100 μl 2 M H_2SO_4. Absorption at 450 nm was measured on a Dynatech Minreader 11.

STANDARD CURVE

A range of dilutions of *V. anguillarum* (strain 8587) in sterile saline were prepared to give optical densities at 610 nm from 0·2–1·0, and each dilution was plated onto TSA for estimation of cfu. An O.D. of 0·38 corresponded to a bacterial concentration of 10^8 cfu ml^{-1}. This value was verified by counting bacterial numbers in a sample of O.D. using a haemocytometer. This gave a value of approx 8×10^7 bacterial ml^{-1}.

PREPARATION OF HEAT-KILLED AND FORMALISED VACCINE

Two batches of TSB without salt (500 ml) were incubated with a culture of *V. anguillarum*, strain 8587. After incubation for 48 h at 22° C the cultures were heat-killed (60° C, 1 h) or were formalised by adding 1% formalin and leaving for 24 h at room temperature.

HATCHING OF CYSTS

Artemia cysts were supplied by Artemia systems NV, Ghent. They were hatched in 30 l diluted seawater (15%) in 80l conical polypropylene tanks, with illumination and vigorous aeration. The temperature was maintained at 30° C and cysts were added at 0·5 gl^{-1}. Nauplii were harvested after 24 h by siphoning into a 200 nm filter and were separated twice to remove unhatched cysts and egg cases. The nauplii were then washed with tap water.

ENCAPSULATION OF VACCINE BY NAUPLII

In order to maximise vaccine ingestion by *Artemia*, simple screening tests were run with two local stages. Instar II metanauplii were used because this corresponds to the first feeding stage, and because fish larvae of different stages can feed on small *Artemia* while they cannot ingest older ones. The procedures adopted were similar to those used for nutrient bioencapsulation in *Artemia* nauplii (Leger *et al.*, 1987). Bioencapsulation of vaccines in instar II and 7-day-old *Artemia* was operated in order to verify the effect of those two different stages on the vaccine's antigenicity. Vaccine concentration and larval densities were varied.

Killed bacteria in the *V. anguillarum* vaccine suspension were stained with Sudan Black III or methylene blue to facilitate their usual inspection once ingested by the *Artemia*.

Microscopic inspection was used for estimation of ingestion levels of vaccine (see Results).

For further study the following procedure was adopted: Instar II nauplii were incubated with Coopers Fiskevax® Vet, batch VA 795/1 or with vaccine produced in the laboratory (formalised). The nauplii were incubated in 1 l of 15% seawater in a conical flask at 30° C with aeration and illumination. The

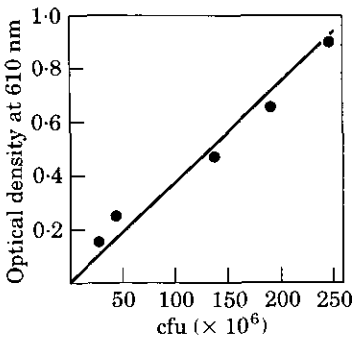

Fig. 1. Standard curve for *Vibrio anguillarum*, strain 8587.

concentration of nauplii was adjusted to 1500 ml^{-1}, unless otherwise stated. Experiments were carried out to determine the time course of vaccine uptake for two formalised vaccine concentrations ($1{\cdot}5 \times 10^7$ and $1{\cdot}5 \times 10^6$ cells ml^{-1}). Samples (30 ml) were removed at various times and the *Artemia* were processed as described below, prior to testing in the ELISA. The uptake of heat-killed versus formalised vaccine was also determined at doses of $1{\cdot}7 \times 10^7$ and $1{\cdot}3 \times 10^7$ cells ml^{-1}, respectively.

OPTIMISATION OF EXTRACTION BUFFER

A variety of extraction buffers and procedures were compared to maximise recovery of antigen from *Artemia*. In the first experiment the extraction of antigen from *Artemia* samples which had been incubated with vaccine for 60 min was compared after 30 min extraction in 0·1% Tween in PBS, 0·1% SDS in PBS or 5 mm DTT in PBS. The effects of the detergents and also of the presence of *Artemia* on the assay were examined by spiking samples with 10^6 ml^{-1} bacteria (*V. anguillarum*, strain 8587).

PREPARATION OF SAMPLES FOR ELISA

Artemia samples were filtered through a 200 nm filter and then thoroughly washed under running tap water. Samples were blotted with tissue paper held under the filter to remove excess water. Samples (10 mg) were weighed and then transferred into a mortar. The samples were ground with PBS/Tween/SDS and then extracted for 30 min before adding the supernatant to the ELISA plate (100 μl).

III. Results

Artemia were shown to take up vaccine from an incubation medium at a level which could be detected by ELISA. A standard curve for *V. anguillarum* strain 8587 cfu *v.* O.D. is shown in Fig. 1. An O.D. of 0·38 represents 1×10^8 cfu ml^{-1}. This value was then utilised to determine bacterial concentrations for standards in an ELISA.

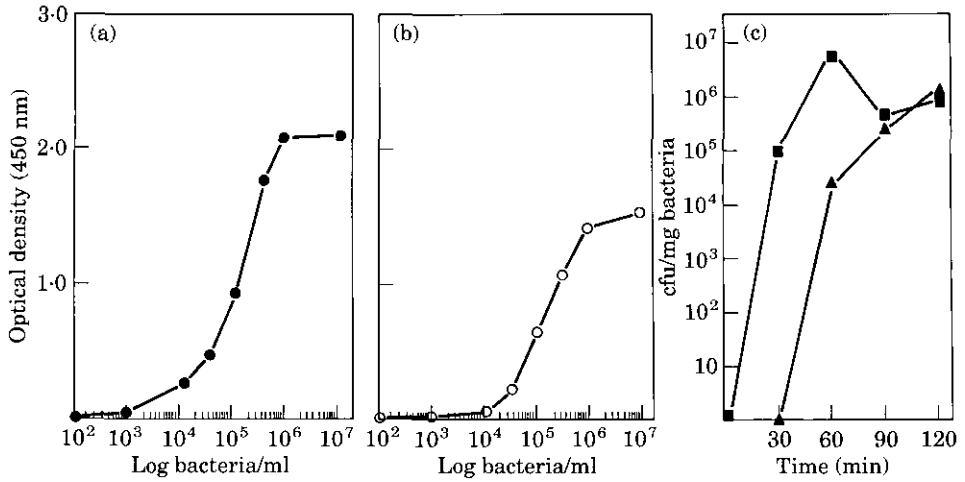

Fig. 2. (a) Standard curve for ELISA of *Vibrio anguillarum*, strain 8587 for high dose vaccine ($1{\cdot}5 \times 10^7$ cfu ml^{-1}). (b) Standard curve for ELISA of *Vibrio anguillarum* strain 8587 for low dose vaccine ($1{\cdot}5 \times 10^6$ cfu ml^{-1}). (c) Time course of uptake of formalised vaccine at two dose rates from an incubation with *Artemia*: (■) high dose ($1{\cdot}5 \times 10^7$ cfu ml^{-1}), (▲) low dose ($1{\cdot}5 \times 10^6$ cfu ml^{-1}).

The optimum larval density for maximum antigen uptake was found to be 1500 ml^{-1}, with vaccine added at $1{\cdot}5 \times 10^7$ cfu ml^{-1}. It was found that the same quantity of vaccine delivered through older *Artemia* can be delivered through a large number of younger ones. Bacterial platings of *Artemia* nauplii from washed and unwashed bacteria were compared. No difference in plates was observed (M. Dehasque, pers. comm.).

The assay was optimised with reference to buffers, detergents and size of *Artemia* sample extracted. Maximum antigen levels were detected after extracton with PBS/SDS. However, spiking PBS/SDS with 10^6 ml^{-1} bacteria resulted in reduced detection of bacteria. Comparing detection of bacterial standards in PBS/Tween and PBS/SDS showed that the presence of SDS caused inhibition of the assay. However, it was found that this could be largely overcome by adding 0·1% Tween to the PBS/SDS. Comparison of PBS/Tween with PBS/Tween/SDS demonstrated an inhibition of the assay by SDS, resulting in a 40% reduction of the values.

Artemia alone in PBS/SDS/Tween gave no reaction in the ELISA. When untreated *Artemia* were spiked with 10^6 ml^{-1} bacteria values were reduced by 22% compared with bacteria in buffer alone. It was therefore necessary to make two adjustments in calculations of antigen levels detected by the assay.

The effect of boiling samples during extraction, and of extending the extraction time was examined. No increase in antigen detection was apparent.

The rate of uptake of antigen from formalised *Vibrio* vaccine was compared at two dose levels. At the higher vaccine dose of $1{\cdot}5 \times 10^7$ cfu ml^{-1}, vaccine was taken up by the *Artemia* at a level of 10^5 cfu mg^{-1} *Artemia* within 30 min, while bacteria were undetectable in *Artemia* at this stage when the low vaccine dose was used ($1{\cdot}5 \times 10^6$ cfu ml^{-1}) [Fig. 2(a)–(c)]. There was a peak in uptake at 60 min with the high vaccine dose, after which it dipped and then

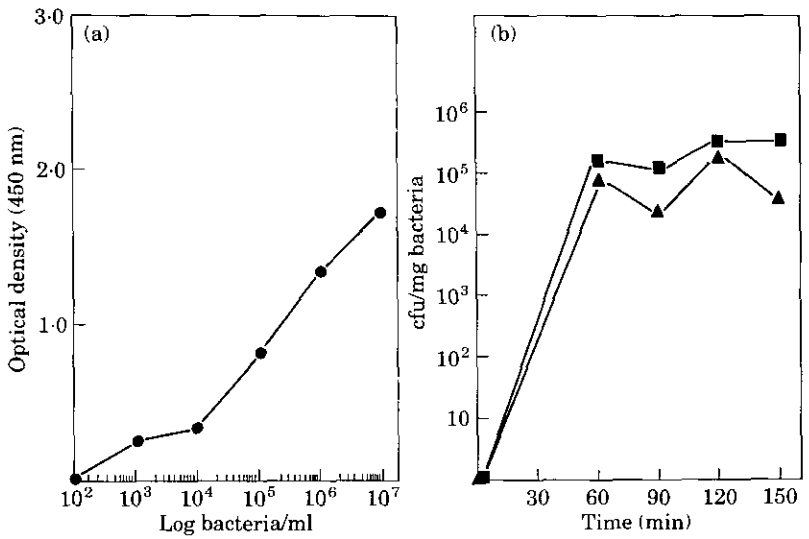

Fig. 3. (a) Standard curve for ELISA of *Vibrio anguillarum* strain 8587 for comparison of heat treated and formalised vaccine. (b) Time course uptake of formalised and heat treated vaccines from incubations with *Artemia* (■) formalised vaccine ($1{\cdot}7 \times 10^7$ cfu ml^{-1}), (▲) heat treated vaccine ($1{\cdot}3 \times 10^7$ cfu ml^{-1}).

levelled out. Further time courses were performed extending the time course up to 360 min (results not shown),. The results were always very similar—a peak in uptake at 60 min followed by a levelling off with a steady state maintained for the rest of the incubation. It appeared that uptake of *Artemia* was maximal at around 5×10^6 cfu mg^{-1} *Artemia*.

The time courses of vaccine uptake from two vaccine formulations were compared [Fig. 3(a) and (b)]. Uptake of antigen from the formalised vaccine was greater than from the heat-treated. There is no evidence that *Artemia* are affected by the presence of 1% formalin in the vaccine formulation, as might be expected.

IV. Discussion

Artemia have been shown to take up vaccine from an incubation medium at a level which could be determined by ELISA. The rate of uptake was concentration dependent, maximum uptake being achieved after 60 min incubation with formalised vaccine at a concentration of $1{\cdot}5 \times 10^7$ cfu ml^{-1} [Fig. 2(a)–(c)]. In a similar study, Kawai *et al.* (1989) investigated the time course of uptake of a vibriosis vaccine by the rotifer, *Brachionus plicantilis*. Uptake was maximal after 30 min incubation at high vaccine concentrations (1400 μg m^{-1}) and was slower at lower concentrations (i.e. μg ml^{-1} and below). Interestingly, if the water fleas were removed from the vaccine and placed in water, the level of antigen dropped rapidly. Antigen levels, expressed at μg lipopolysaccharide g^{-1} water flea, reached 260 μg^{-1} after 30 min incubation and remained at this level for 3 h, but when the fleas were transferred to water, the level dropped rapidly till no antigen was detectable.

This suggests that the antigen is subject to turnover. As it may be altered in this process, it is probably important to keep incubation times as short as possible.

In this study maximum uptake of vaccine was reached when *Artemia* had taken up approx 1×10^5 cfu *V.anguillarium* at the time of testing. This represents only a small percentage of the available antigen (~0·06%) and may indicate that the *Artemia* became 'saturated' with bacteria. As it is not practical to increase the concentration of *Artemia* in the incubation medium, due to physical damage resulting from overcrowding, it is important to establish whether it is possible for a fish to ingest enough vaccine from the treated *Artemia* to achieve protection. A turbot fry (*Scophthalmus maximus*) consumes around 1000 *Artemia* per day (pers. comm. Neil Murdoch, Golden Sea Produce). This would represent a dose of 1×10^8 cfu/day.

Anders (1978) investigated oral immunisation of rainbow trout by feeding formalin-treated freeze-dried *Vibrio* vaccine bound to pulverised pig spleen. A dose of 10^{12} cfu fish^{-1} resulted in an RPS of 77%, while doses of 10^{11} and below resulted in higher mortalities in treated than in control fish.

Agius *et al.* (1983) fed rainbow trout with vaccine-treated food and achieved an RPS of up to 70% with a dose of 10^{12} bacteria/100 g fish. Kawai & Kusada (1985) reported effective immunisation against vibriosis when adult or immature ayu were given a dose of 3×10^9 cfu fish^{-1}, daily, for 8–14 days. These results suggest that a dose of 10^8 cfu fish^{-1} may be too low to produce immunity, but this needs to be confirmed by carrying out a feeding experiment and challenge. Direct immersion vaccination also involves low levels of antigen being sequestered (0·02% of initial bath concentration) (Tatner & Horne, 1983) with the majority being found in the gut (Tatner, 1987). Hence once correct feeding regimes have been established to deliver a similar amount of the antigen to the intestinal epithelium similar protection levels to those afforded by direct immersion should be achievable.

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